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Determination of median levels of the free β subunit of human chorionic gonadotropin in women from mainland China using a new time-resolved fluoroimmunoassay

Yong-Ping Tang[1], Ying-Song Wu[1], Ai-Hua Yin[2], Wei-Wen Xu[1], Peng-Hui Yuan[3] and Ming Li[1,*]

[1] School of Biotechnology, Southern Medical University, Guangzhou, Guangdong Province, P.R. China
[2] Prenatal Diagnosis Center, Guangdong Women and Children Hospital and Health Institute, Guangzhou, Guangdong Province, P.R. China
[3] Guangzhou Darui Antibody Engineering and Technology Co., Ltd, Guangzhou, Guangdong Province, P.R. China

Abstract

Background: The free β subunit of human chorionic gonadotropin (free β-hCG) is an important serum marker for biochemical screening. Its weekly median value varies with ethnicity. Most of the fluorometers for lanthanide chelates are designed for the detection of signals from europium (Eu^{3+}) chelates only.
Methods: We developed a two-site, one-step assay using two monoclonal antibodies (MAbs) against free β subunit and β subunit with Eu^{3+} chelates as labels. Using the present assay, we evaluated 24,634 normal serum samples in Chinese pregnant women during 8–20 weeks of gestation.
Results: The detection limit using this assay was <0.05 ng/mL. The within-run and between-run imprecision was $<6.0\%$ and 7.0% using control material. Free β-hCG concentrations measured using the current assay in 999 maternal serum samples correlated well with those obtained by samarium (Sm^{3+})-labeled DELFIA free hCGβ assay ($r=0.987$). The medians for 8–20 weeks for maternal serum free β-hCG were higher in the women from mainland China compared to reports from other countries.
Conclusions: The present assay is suitable for use in biochemical screening of women in mainland China. Our study on the median concentrations of free β-hCG will help establish reference values that are specific for ethnic populations from the Chinese mainland. These will be useful for studying the importance of ethnic factors in biochemical screening.
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*Corresponding author: Prof. Ming Li, School of Biotechnology, Southern Medical University, 1838 Northern Guangzhou Ave, Guangzhou, 510515, P.R. China
Phone/Fax: +86 20 6164 8550,
E-mail: mingli2006_2009@126.com
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Introduction

As a glycoprotein hormone, there are several forms of human chorionic gonadotropin (hCG) in human serum (1). The free β subunit of human chorionic gonadotropin (free β-hCG) is one of the most important serum markers used in detecting and monitoring pregnancy during the first and second trimesters (2–6). Measurement of free β-hCG has been reported to improve the performance of screening for Down syndrome (DS) (2, 6–9). The concentrations of weekly median values of free β-hCG in pregnancy have been found to be variable according to race or ethnicity (2, 10, 11).

Many laboratories have developed various immunoassay formats for detecting free β-hCG (12–16). PerkinElmer has a commercial kit available (AutoDELFIA hAFP/hCGβ Dual kit and Free hCGβ DELFIA) which are CE approved. These are based on time-resolved fluoroimmunoassay (TRFIA) and are widely used in measurement of free β-hCG in prenatal screening (2, 17–19). Pettersson et al. (14) reported a simultaneous assay for detection of α-fetoprotein and free β-hCG. In these assays, samarium (Sm^{3+}) chelates were used to detect free β-hCG.

In mainland China, three types of fluorometers for detection of lanthanide chelates are available. These include DR-M from Guangzhou Darui Antibody Engineering and Technology Co., Ltd, Any test from Sym-Bio Lifescience Co., Ltd, and TALENT from Guangzhou Fenghua Bioengineering Co., Ltd. However, these instruments can detect signals from europium (Eu^{3+}) chelates only, not from Sm^{3+} chelates that are used for AutoDELFIA hAFP/hCGβ Dual kit and the Free hCGβ DELFIA kit. The objective of this study was to develop and validate a sensitive and specific assay for the detection of free β-hCG in serum using instruments available and produced in China. Eu^{3+} chelates were used to label a specific antibody against β-hCG. After free β-hCG was captured onto the surface of microtitration strips by anti-free β-hCG antibody, the bound fractions of the label were dissociated in a fluorescence enhancement solution, followed by detection with a fluorometer.

Previous studies have shown that the concentration of serum markers in different populations might vary and affect the accuracy of prenatal screening (2, 11). Although several studies have determined differences in free β-hCG concentrations in different populations, the weekly median values determined in these studies are based on small numbers of

cases. The weekly median values of free β-hCG in the second trimester of pregnancy for women from mainland China have never been determined. To obtain more accurate weekly median values for this population, 24,634 serum samples were collected from mainland Chinese women during 8–20 weeks of gestation. Free hCG concentrations were measured using a TRFIA method developed by us. Weekly median values of free β-hCG in these Chinese women were established.

Materials and methods

Materials

Monoclonal antibodies (MAbs) were used for capture and detection. In the two-site assay for free β-hCG, the capture antibody recognizes an epitope present only on the free subunit. For detection, another MAb was used to recognize the β subunit of hCG. MAbs 5004, 5006, 5008 and 5012 were obtained from Medix Biochemica. MAb E82583M was obtained from Meridian Life Science, Inc. MAb ab36212 was obtained from abCAM, Inc. All MAbs were of the IgG_1 class.

Highly purified β-hCG standard was obtained from Meridian Life Science, Inc. The calibrators were calibrated against WHO IRP75/551 for β-hCG (WHO International Laboratory for Biological Standards, Statens Serum Institute). One international unit (1 IU) of the β-hCG standard corresponds to 1 μg. The controls were Lyphochek Maternal Serum Controls and obtained from Bio-Rad Laboratories, Inc. (Hercules, CA, USA).

DELFIA Eu-Labeling kits were obtained from PerkinElmer Life and Analytical Sciences, Inc (PerkinElmer, Waltham, MA, USA). Sephadex G-50 was obtained from Amersham Pharmacia Biotech (Piscataway, USA).

The standard diluent was composed of 50 mmol/L Tris-HCl buffer, pH 7.8, 8.5 g/L NaCl, 1.5% bovine serum albumin (BSA), 0.01% Procline 300 and 0.01% Tween 20. The assay buffer consisted of 50 mmol/L Tris-HCl buffer, pH 7.8, 8.5 g/L NaCl, 0.2% BSA, 0.01% Procline 300, 0.05% bovine globulin, 0.01% Tween 20 and 0.02% diethylene triamine penta-acetic acid.

The DELFIA Free hCGβ kits (A097-101) and enhancement solution used to develop the fluorescence were also from PerkinElmer. The serum specimens were selected from normal pregnancies, and ranged from gestational week 8–20. Samples were obtained from Guangdong Women and Children Hospital and Health Institute of China. Polystyrene microtiter strips were purchased from Labsystems Oy (Helsinki, Finland). The study was approved by the Ethical Committee of the Southern Medical University, and it complies with current ethical considerations.

Instruments

For spectral analysis of the fluorescent chelates, a 1420 Multilabel Counter (Victor^{3}TM) from PerkinElmer was used with filters for Eu^{3+} (613 nm) and Sm^{3+} (643 nm).

Immobilization of capture antibody

Polystyrene microtiter strips were coated with capture antibody using physical adsorption. One hundred and fifty μL of 50 mmol/L sodium carbonate buffer, pH 9.6, containing 3 μg of purified antibody per microliter, was added to each well and incubated at 30°C for 4 h, followed by 4°C overnight. The strips were washed twice and incubated further overnight at 4°C with 200 μL blocking solution (1 g BSA in 50 mmol/L Tris-HCl plus 30 g of trehalose plus 0.05% Procline 300). The blocking solution was discarded and strips were packed in vacuum foil bags containing a humidifier, and stored at 4°C until use.

Labeling of antibodies with Eu^{3+} chelates

Before labeling, the antibodies were filtered, desalted, and concentrated through a Microcon Centrifugal Filter Device (YM-50). This resulted in a final concentration of ~2 mg/mL. For labeling, 1 mg of protein, 0.2 mg of Eu^{3+}-DTTA [N^1-(p-isothiocyanatobenzyl)-diethylene-triamine-N^1,N^2,N^3,N^4-tetraacetate chelated with Eu^{3+}] was added, mixed gently and incubated overnight at 25°C. Free chelates were separated from labeled proteins using gel filtration on a Sephadex G-50 column (1.5×40 cm).

Antibody selection

According to sandwich assay, six kinds of MAbs against the free subunit (5012, E82583M and ab36212) and against the β subunit (5004, 5006 and 5008) were tested in pairs, with each antibody being used as a capture or detection antibody. A one-step sandwich assay format was used together with a 200-ng/mL β-hCG and a blank solution.

Procedure of the free β-hCG-TRFIA

Twenty-five μL of standard and samples added into each well of coated microtitration strips, followed by 100 μL of assay buffer containing 20 ng Eu^{3+}-labeled detection antibodies. The strips were incubated at room temperature (RT) with continuous shaking. After 1 h, the strips were washed 6 times. Two hundred μL of enhancement solution (cat: B118-100) was then added to each well and the strips were incubated at RT for 5 min with slow shaking. Finally, the fluorescence of each well was measured.

Methods for comparison: free β-hCG Eu^{3+}-labeled assay vs. Sm^{3+}-labeled assay

Nine hundred and ninety-nine maternal serum samples from 8 to 20 weeks gestation were collected. Free β-hCG concentrations were measured using the commercial Sm^{3+}-labeled free hCGβ DELFIA assay, and the current Eu^{3+}-labeled free β-hCG-TRFIA. The two immunoassays were performed simultaneously.

The detection of 8–20 weekly median values

Twenty four thousand six hundred and thirty-four serum samples from maternal screening programs in mainland China during 2006–2007 were measured. Only normal singleton pregnancies were selected. The samples were collected during 8–20 weeks of gestation. Gestational age was based on the last menstrual period (LMP). Lyphochek Maternal Serum Control, levels 1, 2, 3, were analyzed in each assay. Control results met the laboratory criteria for acceptability. Medians and means for serum free β-hCG during different gestational weeks were calculated. The data were converted to multiples of the median (MoM) for normal pregnancies at the relevant gestational age with our established weekly median values.

Statistical methods

Deming regression was performed with the Analyse-it add-in for Microsoft Excel (version 2.12; Analyse-it Software Ltd, Leeds,

UK). Other statistical analyses were performed using SPSS software (version 13.0, SPSS Inc, Chicago, IL, USA). A p-value of <0.05 was considered for statistical significance.

Results

Best antibody pair

Antibody selection was based on the results of antibody combination studies. A good pair should give lower detection limits, a better linear dose-response signal, and lower cross-reactivity. Our results from six MAbs clones showed that the best antibody pair contained 5012 (against the free β subunit) as the capture antibody and 5006 (against the β subunit) as the detection antibody (data not shown).

Kinetics, dilution and specificity

Standards and samples (including six maternal serum samples) were incubated for 0.5, 1, 2, 3 h at RT. For standards and serum samples, equilibrium was reached in 1 h. The results showed that a 1-h incubation at RT was sufficient to give more than 85% of the maximum fluorescence intensity; longer incubation times did not improve the sensitivity. Parallel analysis was performed with five serum samples diluted serially from 1/2 to 1/32 with assay buffer. As shown in Figure 1, all five samples diluted linearly. We also tested the specificity of TRFIA by measuring cross-reactivity of the present assay against luteinizing hormone (LH), follicle stimulating hormone (FSH), hCG and thyroid stimulating hormone (TSH). The results indicated that this assay has a very low cross-reactivity against LH, FSH, TSH and hCG (Table 1).

Calibration curves, assay range, detection limit and imprecision

A typical log calibration curve was linear in the range of 3–200 ng/mL for β-hCG (Figure 2, each point is based on ten replicates). Linear regression analysis showed a slope (SD) of 0.9181 (0.0078), a y-intercept of 4.5196 (0.0124) ng/mL, $r^2=0.9998$ and a $S_{y|x}$ value of 0.0115 ng/mL. The highest fluorescence intensity for 200 ng/mL β-hCG was ~4,300,000 counts per second (CPS). No hook effect was observed within the linear range. The detection limit of this assay was <0.05 ng/mL, corresponding to a minimum detectable dose of 1.5 pg/mL per well. The detection limit was defined as the concentration corresponding to the background counts plus 2 SD (n=20). The within-run imprecision (n=10) of standards was between 2.2% and 3.5%.

Within- and between-run assay variation was determined using three levels of maternal serum controls and the same batch of reagents on different days. The results are summarized in Table 2. The intra- and inter-assay coefficients of variation (CV) ranged from 4.2% to 5.2% and 3.4% to 6.2%, respectively.

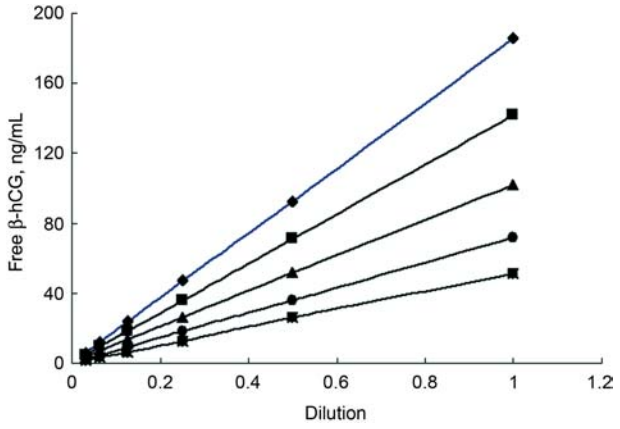

Figure 1 Dilution curves for free β-hCG concentrations based on five serum samples using the Eu^{3+}-labeled TRFIA (2- to 32-fold dilution).

Table 1 Cross-reactivity of various hormones using Eu^{3+}-labeled TRFIA.

Hormone	Concentration, IU/L	Free β-hCG concentration, ng/mL
LH	300	<0.05
FSH	200	<0.05
TSH	0.30	<0.05
hCG	2000	<1.5

Comparison of methods

We measured free β-hCG concentrations in 999 first or second trimester specimens using both the DELFIA Free hCGβ assay and the current free β-hCG-TRFIA. These data were compared using Deming regression [slope (95% CI), 1.0117

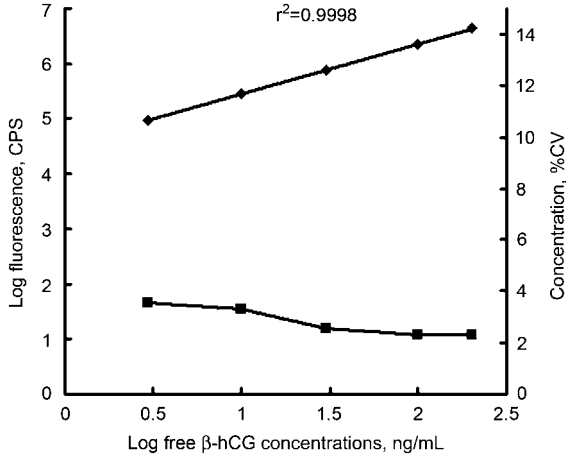

Figure 2 Calibration curve (♦) and corresponding within-assay imprecision (■) profile for free β-hCG.
The background fluorescence of ~1938 CPS has been subtracted. Each point is based on ten replicates and the standard deviations of repeated points were 0.11, 0.33, 0.76, 2.30 and 4.57 ng/mL, respectively.

Table 2 Intra- and inter-assay coefficients of variation (CV) using the Eu^{3+}-labeled TRFIA for measuring free β-hCG.

Serum control	Free β-hCG, ng/mL	Intra-assay CV (%, n=35)	Inter-assay CV (%, n=68)
39,061	20.0	5.2	6.2
39,062	72.9	5.3	4.5
39,063	122.0	4.2	3.4

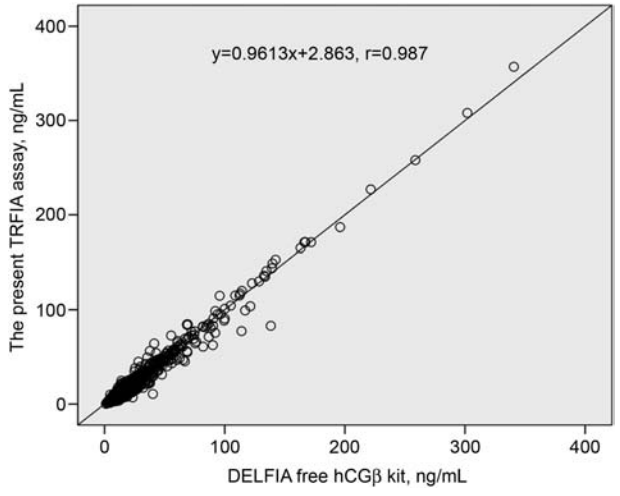

Figure 3 Comparison of free β-hCG concentrations using the present TRFIA and the DELFIA free hCGβ kit measured in 999 maternal serum samples.

(0.9897–1.0337); y-intercept (95% CI), –2.4761 (–2.8986 to –2.0535) ng/mL; $S_{y|x}$, 4.8055 ng/mL]. The two assays showed a correlation coefficient of 0.987 ($p<0.001$) (Figure 3).

Establishing weekly median values in normal singleton pregnancies

By measuring 24,634 samples from mainland Chinese women with the assay we established, we determined the weekly median values for free β-hCG (Table 3). Free β-hCG concentrations at different gestational weeks did not fit a Gaussian distribution. The median values decreased with the increase in gestational week. The average age of women in the maternal serum screening program was 28.05 years.

Discussion

By combining two MAbs against the free β subunit and β subunit of hCG, and using Eu^{3+} as the probe for signal detection, we developed an immunofluorometric assay for free β-hCG with high specificity, very high sensitivity, good reproducibility and short incubation time. Pre-dilution of the sample is not required and measurement of free β-hCG is easy. The analytical performance of the present assay was acceptable and 100 patient samples could be analyzed in 90 min. The fluorescent Eu^{3+} chelates had high quantum yields. However, the Sm^{3+} chelates had lower fluorescence intensity (14, 20, 21). Thus, the signal intensity of our assay with Eu^{3+} was about 60 times higher than the DELFIA Free hCGβ kit. The sensitivity of our assay was <0.05 ng/mL; much lower than that of the DELFIA Free hCGβ (below 0.2 ng/mL, from its user manual) and the method by Pettersson et al. (below 0.2 μg/L) (14). The concentrations of free β-hCG measured in this study showed good agreement with the DELFIA Free hCGβ method from PerkinElmer. Thus, our assay could take the place of the Sm^{3+}-labeling methods for detection of free β-hCG.

As mentioned previously, current fluorometers for lanthanide chelates made in China can detect signals from Eu^{3+}

Table 3 Value of serum free β-hCG during different weeks of gestation.

Gestational week	Number of samples	Free β-hCG, ng/mL Medians (raw data)	Free β-hCG, ng/mL Mean	5th Percentile	95th Percentile
8	52	110.0	127.88	32.76	301.54
9	503	109.0	116.08	37.22	228.60
10	704	83.5	97.29	26.53	228.00
11	1095	65.7	78.68	21.68	183.20
12	1626	58.1	69.78	18.60	161.00
13	2276	47.2	58.63	16.09	139.00
14	1645	30.9	36.28	11.93	75.65
15	2378	22.8	26.84	9.35	54.81
16	3033	17.7	20.73	7.04	43.63
17	3614	13.8	16.55	5.66	34.75
18	3141	11.9	14.00	28.10	1.18
19	2545	10.8	12.34	24.30	1.14
20	2022	9.44	11.06	22.69	1.17

chelates only, and not Sm^{3+}. Therefore, our assay using Eu^{3+} chelates is more suitable for measurement of free β-hCG in mainland China, which should promote prenatal screening in this area.

The median concentrations of free β-hCG were influenced by the ethnic origin of the woman. Kagan et al. (2) described a 12% increase in free β-hCG in women of Afro-Caribbean origin compared with Caucasians. Leung et al. (11) reported that Chinese had significantly higher maternal serum free β-hCG concentrations and pregnancy associated plasma protein-A (PAPP-A), measured in 943 women during the first trimester of pregnancy. Our study was based on 24,634 samples from women from mainland China. We confirmed the difference due to race and showed that median concentrations of serum free β-hCG were higher in Chinese compared with other groups. In our study, the free β-hCG median concentrations in Chinese were 27%–68% higher than those provided with the DELFIA Free hCGβ kit, 39%–124% higher than the results by Krantz et al. (22), 35%–68% higher than those reported by Spencer (23) and 20%–44% higher than results from Pettersson et al. (14). Our study showed that free β-hCG median concentrations in Chinese were higher than found in other studies. In mainland China, PRISCA (Siemens) and 2T-Risks (3) (PerkinElmer) are most widely used for risk assessment. Failure to take into account ethnic differences in Chinese women would result in overestimation of the true risk of trisomy 21, and underestimation of the true risk of trisomy 18. Therefore, our study provides reference data for adjusting free β-hCG medians for biochemical screening in women from mainland China. In addition, our study should attract interest with respect to the effects of ethnic differences on biochemical screening.

In conclusion, the present TRFIA, specific for free β-hCG, provides high sensitivity. This assay is more versatile for meeting the needs of women from mainland China. Our study on the median concentrations of free β-hCG will help to establish reference values specific for the ethnic population of mainland China, and is useful for studying the importance of ethnicity on biochemical screening.

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Conflict of interest statement

Authors' conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article. Research funding played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.
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